INFECTION AND IMMUNITY, Dec. 1990, p. 4149–4152
0019-9567/90/124149-04$02.00/0

Vol. 58, No. 12

Susceptibility of Inbred Mice to *Leishmania major* Infection: Genetic Analysis of Macrophage Activation and Innate Resistance to Disease in Individual Progeny of P/J (Susceptible) and C3H/HeN (Resistant) Mice

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Received 22 June 1990/Accepted 14 September 1990

We tested the possibility that two phenotypic traits, defective activation of macrophage antileishmanial activities and susceptibility to infection with *Leishmania major*, were controlled by the same gene. We used P/J (susceptible) and C3H/HeN (resistant) mice to breed F_1, backcross (Bx), and F_2 mice that were tested individually for both traits, each of which is known to be controlled by a single autosomal gene. We found no correlation between the macrophage defect and cutaneous disease. There was a correlation between development of systemic disease and defective macrophage activation in Bx mice; this correlation, however, was not confirmed in the F_2 population.

The leishmanias are protozoan parasites that infect and replicate only inside macrophages in their mammalian hosts. This intracellular niche presents a unique opportunity for the parasite to evade many humoral host defense mechanisms. The one immunologic response that the parasite cannot evade, however, is the acquisition of potent intracellular killing activities of immunologically stimulated macrophages (9). Thus, a particular host's natural resistance to infection with this parasite may depend upon its ability to activate macrophages for the destruction of intracellular parasites. When inbred mouse strains are analyzed for host response to infection with *Leishmania major*, a parasite that causes a cutaneous disease with systemic sequelae, only a few strains are susceptible (7). The best characterized of the susceptible mouse strains is BALB/c; susceptibility in this strain is very complex and involves, among other things, suppressor T lymphocytes and an imbalance of Th1 and Th2 cells (4, 5, 10). Other susceptible strains include C57L/J, SWR/J, and P/J mice (7). Susceptibility in all strains appears to correlate with a macrophage defect: each strain susceptible to infection is also a strain whose macrophages do not respond in vitro to lymphokine signals for intracellular killing of the parasites (7). To further characterize the genetics of these two phenotypic traits (susceptibility to infection and in vitro macrophage response to lymphokines for intracellular killing) we selected the P/J strain (Jackson Laboratory, Bar Harbor, Maine) as our prototypic susceptible strain and the C3H/HeN strain (Harlan Sprague Dawley, Indianapolis, Ind.) as our resistant strain. From these naturally susceptible and resistant stocks, we bred (C3H/HeN × P/J)F_1, (F_1 × F_1)F_2, and backcross (Bx) (F_1 × P/J) mice in the barrier animal facility at the Walter Reed Army Institute of Research. In studies reported separately, we found that both phenotypes were controlled by single, autosomal, dominant genes (2, 3, 8). Since there was a strong correlation between susceptibility to infection and macrophages unresponsive to lymphokines in all mouse strains tested (7), it was possible that both phenotypic traits were controlled in P/J and C3H/HeN mice by the same gene. To test this possibility, we set up a prospective study. Peritoneal macrophages were obtained from each individual mouse from the parental mouse strains (P/J and C3H/HeN) and their progeny twice at weekly intervals and tested for lymphokine-induced intracellular destruction of *L. major* amastigotes in vitro at each time point. After the second peritoneal cell harvest, each animal was rested for several days and then inoculated subcutaneously with *L. major* amastigotes. At week 12 after infection, footpad depth and number of amastigotes in visceral tissues were assessed. In this way we were able to compare the two phenotypes, in vitro macrophage intracellular killing activities and in vivo resistance to infection, in individual mice and analyze the correlation between these two traits.

Peritoneal cells were obtained from individual animals before infection by inoculation of 5 ml of warm RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 1% heat-inactivated fetal bovine serum through a 27-gauge needle at the top of the peritoneum. Fluid was collected through a 19-gauge needle at the base of the peritoneum. The mice were earmarked for future identification. The animals were rested for 7 days, and the procedure was repeated. The average yield of macrophages from a single animal by this procedure was 2×10^6/day. Each day, macrophages were prepared for the intracellular killing assay by procedures that are standard in our laboratory (9). Peritoneal cells (50% macrophages) were cultured as cell pellets in polypropylene tubes. *L. major* (World Health Organization strain WHOM/IR/−/173) used in the in vitro killing assay was propagated in footpads of BALB/cJ mice (Jackson Laboratory). Amastigote suspensions were prepared from footpads 3 to 4 weeks after inoculation by standard procedures (9). Viable parasites were estimated by hemacytometer counting of fluorescein diacetate-ethidium bromide-stained samples (6). Macrophages were infected with one amastigote per macrophage and treated immediately with lymphokines. Lymphokines were generated from C3H/HeN mice immunized intradermally with viable *Mycobacterium bovis* BCG (10^6 CFU). Three to six weeks after BCG immunization, spleens were aseptically removed and lymphokine-containing spleen cell culture fluids were prepared as described previously (9).

TABLE 1. Macrophage activation, cutaneous disease, and systemic disease in individual Bx mice [(C3H/HeN × P/J)F_1 × P/J]

% Microbicidal activity	Rank	Footpad depth (mm)[a]	Rank	Systemic disease (LDU)[b]	Rank
95	1	0.7	17.5	0	14.5
92	2	0.7	17.5	0	14.5
89	3	2.5	29	0	14.5
84	4	1.5	23	0	14.5
74	5	1.6	25.5	0	14.5
70	6	0.4	11	0	14.5
67	7	0.4	11	0	14.5
65	8	1.8	28	0	14.5
59	9	D	33.5	>1,000	34
45	10.5	0.5	14.5	0	14.5
45	10.5	0.3	7	0	14.5
43	12	—	33.5	201	31
41	13	0.2	4.5	0	14.5
36	14	0.3	7	0	14.5
34	15	0.5	14.5	0	14.5
31	16	1.6	25.5	0	14.5
26	17	0.9	19	0	14.5
25	18	4.4	31	448	32
22	19	1.7	27	3	30
18	20	4.1	30	0	14.5
17	21	0	1.5	0	14.5
14	22	1.2	20.5	0	14.5
12	23	0	1.5	0	14.5
10	24	1.2	20.5	0	14.5
8	25	0.2	4.5	0	14.5
1	26	0.6	16	0	14.5
0	31	0.3	7	0	14.5
0	31	0.4	11	0	14.5
0	31	1.5	23	0	14.5
0	31	0.1	3	0	14.5
0	31	0.4	11	0	14.5
0	31	1.5	23	0	14.5
0	31	D	33.5	>1,000	34
0	31	D	33.5	>1,000	34
0	31	0.4	11	0	14.5

[a] —, Animal lost the infected foot to necrosis; D, animal died before 12 weeks.

[b] Livers were harvested at 12 weeks for determination of LDU except for animals that died before 12 weeks; livers were biopsied within 2 days of death.

Control supernatants consisted of the culture media from spleen cells of BCG-infected animals, to which purified protein derivative was added after 48 h of incubation. These sham lymphokine fluids did not induce microbicidal activity in the lymphokine-responsive C3H/HeN macrophage cultures. Peritoneal cells were incubated for 72 h at 37°C in 5% CO_2 in moist air. The percentage of macrophages with intracellular amastigotes was determined by microscopic examination of stained cell smears at 72 h. Results were expressed as mean percentages of leishmania-infected macrophages for four different observations of one or two cultures (400 to 800 macrophages observed). Microbicidal activity was defined as the percent decrease in infected macrophages in lymphokine-treated cultures compared with medium-treated controls for each animal and was determined by the following formula:

$$\frac{(\%\text{ infected control macrophages} - \%\text{ infected treated macrophages}) \times 100}{\%\text{ infected control macrophages}}$$

The data in Table 1 are the means of two in vitro assays on peritoneal cells from each animal in the Bx population; the data in Table 2 are from the F_2 population. Variability was never >20% and was usually <10%. P/J mouse macrophages tested simultaneously for response to lymphokine for intracellular destruction of *L. major* had a mean response of 5.8%, with a standard deviation of 10%. Mice from the Bx and F_2 populations were characterized as responsive if the macrophage response from these animals was greater than two standard deviations above the mean P/J mouse macrophage response (5.8% + 20%, or 26%). Chi-square (χ^2) analyses were performed on these data to confirm previous results that indicated single-gene control of macrophage response to lymphokine for intracellular killing of parasites (3). On the basis of this criterion, analysis of the entire population of Bx mice (35 mice in this study and 52 mice used in our previous study [8]) for a difference between expected results (single-gene control of macrophage response to lymphokine for intracellular killing) and observed results determined that 46 of 87 mice were responsive (52.8%). Statistical analysis gave a χ^2 value of 0.18 ($P > 0.1$),

TABLE 2. Macrophage activation, cutaneous disease, and systemic disease in individual mice of the (F_1 × F_1)F_2 population

% Microbicidal activity	Rank	Footpad depth (mm)[a]	Rank	Systemic disease (LDU)[b]	Rank
92	1	0.3	10.5	0	15.5
86	2.5	0.2	8	0	15.5
86	2.5	0.3	10.5	0	15.5
84	4	0.4	12	0	15.5
83	5	3.9	34	179	36
78	6.5	3.4	33	340	37
78	6.5	0.7	15.5	0	15.5
77	8	0.9	19	0	15.5
75	9	1.0	22	0	15.5
73	10	0	3	0	15.5
72	11.5	3.2	32	0	15.5
72	11.5	—	38	80	35
64	13	0.5	13	0	15.5
61	14	2.8	31	0	15.5
60	15	2.3	28	35	33
57	16	1.1	24	0	15.5
52	17	0	3	0	15.5
46	18	2.6	29	0	15.5
41	20	—	38	0	15.5
41	20	—	38	916	38
41	20	D	38	>1,000	39.5
34	22	1.0	22	0	15.5
32	23.5	2.7	30	0	15.5
32	23.5	0	3	0	15.5
26	25	0.8	17	0	15.5
21	26	1.6	26	57	34
20	27	0.6	14	0	15.5
17	28	6.4	35	0	15.5
12	29	0	3	0	15.5
9	30.5	0.2	8	0	15.5
9	30.5	0.7	15.5	0	15.5
8	32.5	D	38	>1,000	39.5
8	32.5	1.7	27	0	15.5
4	34	0	3	0	15.5
3	35	0.9	19	0	15.5
1	36	0.9	19	0	15.5
0	38.5	0.1	6	0	15.5
0	38.5	0.2	8	0	15.5
0	38.5	1.5	25	0	15.5
0	38.5	1.0	22	0	15.5

[a] —, Animal lost the infected foot to necrosis; D, animal died before 12 weeks.

[b] Livers were harvested at 12 weeks for determination of LDU except for animals that died before 12 weeks; livers were biopsied within 2 days of death.

and the expanded number of Bx mice confirmed previous determinations of single-gene control of this trait. In this particular study, 17 of 35 (48.5%) Bx mice were macrophage responsive, with a χ^2 value of 0.014 ($P > 0.1$). Even in the present study, the ability of macrophages from Bx progeny to become activated for intracellular destruction of the parasite is due to a single gene. The F_2 population was not used to confirm the data in the previous study (8), but analysis of the mice in the present study also confirmed the results seen with the Bx mice, albeit with a small sample size (40 mice). Twenty-five of 40 (62.5%) F_2 mice had lymphokine-responsive macrophages, with a χ^2 value of 0.8 ($P > 0.1$). Thus, for in vitro macrophage response to lymphokines for intracellular killing, the observed results are not different from those that would be expected if this response were controlled by a single gene.

Seven days after the last peritoneal cell harvest, we inoculated each mouse in the left rear footpad with 50 μl of an *L. major* amastigote suspension that contained 25×10^5 viable parasites. This concentration reliably induces fulminating cutaneous and systemic disease in BALB/c mice, the standard susceptible strain, in the 12-week observation period. The contralateral footpad received an equal volume of medium. Footpad depths were measured with Schnell-taster calipers (Carobronze Ltd., London, England), and lesion size was determined by subtracting the medium-inoculated footpad depth from the *L. major*-inoculated footpad depth. The criterion for determination of resistance to cutaneous infection was determined from the mean + two standard deviations (0.3 + 0.6 mm) of the footpad depth of resistant C3H/HeN mice inoculated with *L. major* during these experiments. Thus, mice that developed footpad lesions ≤0.9 mm deep 12 weeks after inoculation of *L. major* were considered resistant. When all of the data for Bx mice in this experiment and previous experiments were analyzed (35 mice from this study and 76 mice from the previous study [2]), 64 of 111 animals were resistant. If cutaneous disease were controlled by a single gene, then 50% of the Bx mice would be expected to be resistant. When the data for the 111 Bx mice are statistically analyzed for a difference between expected and observed results, the χ^2 value is 1.3, which corresponds to a P of >0.1. Thus, the observed percentage of resistant Bx mice (57%) is not significantly different from the percentage that would be expected if control were by a single gene. In this particular experiment, 19 of 35 (54%) Bx mice were resistant, with a χ^2 value of 0.13 ($P > 0.1$). Chi-square analysis of the entire F_2 population (94 mice from previous experiments [2] and 40 mice from this study) yields a χ^2 value of 3.04 ($P > 0.05$); the observed results with all tested mice are not different from those expected for single gene control. In the F_2 population of the present study, 20 of 40 animals were characterized as resistant. Because of the small number of mice, the χ^2 value for this population is 3.3 ($P > 0.05$). These data, as above, confirm and extend the results of the original paper (2) that describes single-gene control of development of cutaneous disease due to *L. major*.

To analyze systemic disease in *L. major*-infected mice, we sacrificed animals by decapitation 12 weeks after footpad inoculation. The livers were excised aseptically and weighed, and the numbers of amastigotes per 1,000 nucleated cells were counted in organ impression smears stained with Wright-Giemsa stain. This number was multiplied by the organ weight to give an estimation of the organ parasite load and was expressed as Leishman-Donovan units (LDU). The footpad depths (in millimeters) and LDU at 12 weeks for each individual in the Bx and F_2 populations are also shown in Tables 1 and 2, respectively. The development of systemic disease has not previously been examined by classical genetic analysis with these two parental strains.

We ranked the data from three different observations of each mouse: (i) in vitro lymphokine-induced macrophage antimicrobial activity, (ii) size of footpad lesion at 12 weeks, and (iii) number of LDU, as a measure of systemic disease, at 12 weeks. Assigned ranks are contained in Tables 1 and 2. Ranking of data was necessary, since we were analyzing discontinuous and continuous data as well as discrete and nominal variables. The most desirable values were given a low rank. For example, the highest microbicidal activity in response to lymphokine, the lowest footpad depth 12 weeks after infection with *L. major*, and the smallest number of parasites found in visceral tissues were all given a rank of 1 within each population. For cutaneous disease, the larger the footpad depth, the higher the rank; lesions with necrotic tissue received a higher rank than nonnecrotic lesions, and death received the highest rank. The ranks were then used in a Spearman rank coefficient analysis of correlation between either (i) in vitro macrophage response to lymphokines and in vivo development of cutaneous disease or (ii) in vitro macrophage response to lymphokines and in vivo development of systemic disease. The Spearman rank correlation coefficient (r_s) is defined as follows:

$$r_s = 1 - [6\Sigma d^2/n(n^2 - 1)]$$

where d is the difference between the ranks of paired observations and n is the population size. The rank correlation coefficient varies from −1 to +1; 0 indicates no correlation, +1 indicates a positive correlation, and −1 indicates an inverse correlation. The critical values for rejection of the null hypothesis (that the two variables in question are not correlated) were determined for each population and used as criteria to either accept or reject the null hypothesis. We compared the computed r_s with the critical values (the minimum r_s value required) for rejection of the null hypothesis. Critical values were obtained from standard statistical tables (1) as follows: 0.33 for the Bx population ($n = 35$) and 0.31 for the F_2 population ($n = 40$) at the 0.05 level of significance, and 0.42 for the Bx population and 0.39 for the F_2 population at the 0.01 level of significance. The computed r_s for the correlation of footpad lesions (cutaneous disease) with in vitro macrophage response to lymphokine for intracellular killing in the Bx population was −0.14; for the F_2 population it was −0.06. The calculated r_s for the correlation of the number of liver LDU at 12 weeks (a measure of systemic disease) with macrophage response to lymphokine for killing in the Bx population was 0.35; for the F_2 population the r_s was 0.13. In both the Bx and F_2 populations, the correlation coefficient for association between macrophage activation and resistance to cutaneous disease was less than the critical value for rejecting the null hypothesis; thus, these two variables were not correlated. The r_s value for an association between in vitro macrophage activation and systemic disease in the Bx population was 0.35. This value exceeded the critical value needed to reject the null hypothesis at the 0.05 level of significance but not at the 0.01 level. The r_s value for the same association in the F_2 population was only 0.13, a value much lower than the critical value for rejecting the null hypothesis.

Although both macrophage activation for antimicrobial activities and susceptibility to *L. major* infections are controlled by single autosomal dominant genes in P/J and C3H/HeN mice (2, 3, 8), analysis of these two traits in a

single animal demonstrates conclusively that the genes segregated in progeny of the two parental strains. There was no correlation between macrophage intracellular killing capacity and development of cutaneous lesions; there was a tenuous correlation between development of systemic disease and defective macrophage activation in Bx mice that could not be substantiated in the F_2 population. Thus, defective macrophage response to lymphokines for intracellular destruction of *L. major* amastigotes was not the proximal cause of susceptibility in the P/J mice. The nature of the genetic defect that renders P/J mice susceptible to the lethal effects of *L. major* remains a mystery.

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